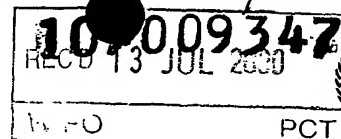




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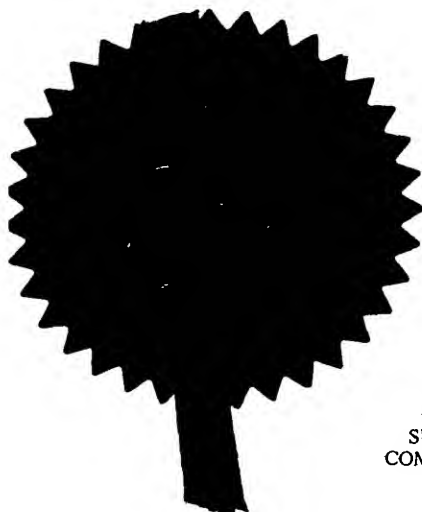
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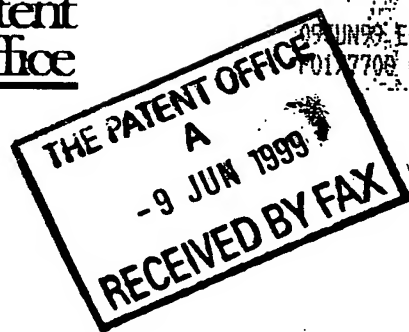
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03984150003

4. Title of the invention

POLYMER MODIFIED BIOLOGICAL ELEMENTS

5. Name of your agent (if you have one)

"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

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DUPLICATE

POLYMER MODIFIED BIOLOGICAL ELEMENTS

The present invention relates to polymer modified viruses and micro-organisms, processes for their preparation and their use in various biotechnology strategies including agriculture and medicine.

5 Background

Micro-organisms and viruses find many applications throughout the broad fields of biotechnology. They are involved in medicine, agriculture, industrial production processes (including notably the oil and brewing industries) and bioremediation. Many useful applications and functions have
10 been identified and developed for such biological agents. However, often the development or enhancement of their activities is limited by their precise properties, restricting their ability to fulfil tasks that are theoretically possible but practically beyond their scope. In this situation, which is commonly encountered, it is desirable to re-engineer the properties of the virus or micro-
15 organism to endow it with properties more appropriate for its required purpose.

For example biological insecticides such as baculoviruses may be restricted in their usefulness through inappropriate target specificity; sulphur metabolising bacteria may be limited in their useful application in the petrochemical industry through inadequate patterns of dispersion and
20 distribution; and in the context of human or veterinary gene therapy, viruses intended to mediate delivery of therapeutic genes may be limited in their usefulness through inefficiency of transgene expression in target tissues.

The field of somatic cell gene therapy has attracted major interest in recent years because it promises to improve treatment for many different types
25 of disease, including both genetic diseases (e.g. cystic fibrosis, muscular dystrophy, enzyme deficiencies) and diseases resulting from age- or damage-related physiological deterioration (cancer, heart disease, mature onset diabetics). However although the field has seen rapid and extensive development, including initiation of over 100 clinical trials, instances of clear
30 therapeutic benefit to patients are very few. One antisense technology has recently been licensed for human use, but no gene therapy strategies have fulfilled their original promise and none are likely to be approved for routine clinical application in the foreseeable future.

The reasons for lack of therapeutic efficacy partly reflect the patient
35 population (most patients enrolled for these experimental treatments are

relatively sick, and even an effective treatment might show little therapeutic benefit) but primarily reflect the inadequate levels, duration and distribution of expression of therapeutic genes achieved. In short, the successful application of sophisticated treatment strategies is limited by inadequate vectors for gene delivery and expression.

Two main types of vectors have been explored so far – either non-viral (usually based on cationic liposomes) or viral (usually retroviruses, adenoviruses, latterly adeno-associated viruses (aav) and lentiviruses).

Viruses are the obvious choice as vectors for gene delivery, since this is essentially their sole function in nature. Consequently viruses have seen considerable use in gene therapy to date, forming the majority of vectors employed in clinical studies. The main feature of adenoviruses which limits their successful application is their immunogenicity. Although they are professional pathogens, evolved over millions of years as highly efficient gene delivery vectors, their hosts have similarly developed very effective protection mechanisms. Serum and ascites fluid from cancer patients contain antibodies that can completely prevent viral infection *in vitro* even at high dilution. Typical human protocols involving adenovirus lead to significant inflammatory responses, as well as inefficient infection of target cells.

Although the non-viral systems have a much better safety record, and are easier to produce in large quantities, they have low specific transfection activity and efficiency of gene expression in target tissues has been a major problem.

Another major limitation to successful application of current vectors for treatment of disease is the requirement for their administration directly to the site of disease, either by direct application or by intra-arterial administration. No vectors are capable of targeting to specific cells following intravenous injection. Cationic lipid systems occlude the first capillary bed they encounter, the pulmonary bed, while adenoviruses/retroviruses are rapidly taken up by the liver and (in animal studies) mediate local toxicities. Although local administration can be feasible for treatment of certain diseases (e.g. bronchial epithelial cystic fibrosis), other diseases have a more widespread distribution (notable clinical cancer and atherosclerosis) and intravenous targeted gene delivery is crucial to embrace the possibility of successful gene therapy.

One approach disclosed in WO 98/44143 has been to modify the surface of viruses with mono-functional poly(ethylene-glycol). This can lead to significantly decreased neutralisation of infection by serum antibodies. This approach retains normal receptor-binding and infection (via the CAR receptor for adenovirus). The problem with this is that it does not facilitate re-targeting of the virus to selected receptors to gain useful and therapeutically-relevant tropisms.

WO 98/19710 discloses the use of multivalent polymers to coat cationic complexes of nucleic acid material to act as a carrier vehicle for the material. There is no disclosure or suggestion that the polymers would be useful to coat viruses or other micro-organisms.

One object of the present invention is to provide a virus or micro-organism which is resistant to neutralisation by the host immune system. It is a further object of the invention to be able to target the virus or micro-organism to allow completely flexible tropism, and modification of biological or physical properties.

Summary of the Invention

In accordance with the present invention there is provided a polymer modified biological element wherein the surface of the biological element is linked to a polymer having multiple reactive groups whereby the biological and/or physicochemical properties of said biological element are modified.

A biological element for use in the invention is optionally a virus, a bacteria or a bacteriophage, a fungus, a spore (from fungi or bacteria) or a component thereof containing genetic information.

Preferably the linkage of the polymer to the biological element results in the inhibition of the ability of the biological element to interact with biological molecules with which it normally can interact or in the inhibition of the ability of the biological element to bind to its normal receptors.

Preferably the polymer is linked to the biological element by two or more linkages. It has generally been found that at least two linkages are required to obscure the receptor binding site on the biological element.

According to the present invention there is further provided a process for the preparation of a polymer modified biological element in accordance with the present invention which process comprises combining a biological

element with a polymer. The invention also provides a polymer modified biological element obtainable by this process.

5 The invention further provides a polymer modified biological element for *in vivo* delivery of therapeutic genetic material to a patient in carrying out gene therapy or DNA vaccination treatment for example, wherein the polymer modified biological element is a polymer modified biological element in accordance with the invention comprising a biological element which includes the therapeutic genetic material.

10 According to the present invention there is also provided a method of gene therapy which method comprises administering to a patient in need of such therapy a polymer modified biological element in accordance with the present invention which comprises a biological element which includes therapeutic genetic material.

15 The present invention also provides the use of a polymer modified biological element in accordance with the present invention in the manufacture of a medicament for use in gene therapy wherein the polymer modified biological element comprises a biological element which includes therapeutic genetic material.

20 The present invention further provides a composition comprising a polymer modified biological element in accordance with the present invention in association with a carrier.

25 The present invention provides a polymer modified biological element wherein the polymer is linked to the biological element by two or more linkages. The polymer used in the present invention is in general a multivalent polymer, i.e. it includes multiple reactive groups.

It will be understood that the term "reactive group" is used herein to denote a group that shows significant chemical reactivity, especially in relation to coupling or linking reactions with complementary reactive groups of other molecules.

30 The polymer used in the present invention is preferably a hydrophilic multivalent polymer. The polymer is in general a biologically inert polymer. The polymer backbone is generally substituted by reactive groups. These reactive groups are either connected directly to the polymer backbone or via a spacer group such as an oligopeptide linkage. The oligopeptide linkage is preferably biodegradable and preferably comprises from 1 to 4 peptide groups,
35

especially 2 or 4. Examples of suitable linkages include -Gly-Gly-, -Glu-Lys-Glu-; and -Gly-Phe-Leu-Gly-.

The polymer backbone is preferably based upon monomer units such as N-2-hydroxypropylmethacrylamide (HPMA), N-(2-hydroxyethyl)-l-glutamine (HEG), or ethyleneglycol-oligopeptide. Where the backbone is based upon ethyleneglycol-oligopeptide, the oligopeptide group preferably comprises from 1 to 4 peptide groups. It is the oligopeptide group which is substituted by the reactive group, optionally via a spacer group as defined above.

A suitable reactive group for the polymer is a group which will react with a group present on the surface of the biological element. For example p-nitrophenol (ONp) esters or N-hydroxysuccinimide (NHS) esters could be used.

Examples of suitable polymers for use in the invention are disclosed in WO 98/19710 and include polyHPMA-GlyPheLeuGly-ONp, poly(pEG-lysine(-ONp)), poly(pEG-GluLysGlu(ONp)), pHEG-ONp, pHEG-NHS. The preparation of these compounds is disclosed in WO 98/19710. The contents of WO 98/19710 is included herein by reference.

In some instances the polymer and/or the linkages between it and the biological element are optionally hydrolytically or enzymatically unstable. Hydrolytic instability is desirable since it permits regulation of the time for which the biological element is protected. Thus if the polymer is provided with a tissue-specific targeting group, the polymer (or the linkage between the polymer and the biological element) could be designed so that the polymer protects the biological element for as long as it takes for the modified biological element to reach the appropriate situation within the target tissue before disintegrating, freeing the biological element to interact with the tissue. Alternatively the polymer could be designed to disintegrate at a rate yielding optimal kinetics of release of the biological element. Enzymatic instability is desirable since it permits the polymer (or the linkage between the polymer and the biological element) to be designed for cleavage selectively by chosen enzymes. Such enzymes could be present at the target site, endowing the modified biological element with the possibility of triggered disintegration at the target site, releasing the biological element for interaction with the target tissue. The enzymes may also be intracellular, permitting disintegration of the modified biological element in selected cellular compartments to enhance the activity of the biological element. Alternatively enzyme-cleavage sites may be

designed to promote disintegration of the modified biological element in response to appropriate biological activity (eg. arrival of an invading or metastatic tumour cell expressing metalloproteinase). Finally, enzymes capable of activating the modified biological element may be administered at the appropriate time or site to mediate required disintegration of the modified biological element and subsequent interaction of the biological element with the tissue.

The polymer used to modify the biological element is preferably cross-linked such that it forms a hydrogel. The hydrogel is preferably hydrolytically unstable or is degradable by an enzyme, for example matrix metalloproteinases 2 or 9. This is in order that the biological elements are immobilised within the hydrogel and that the release of the biological elements can be regulated. Thus, according to a preferred feature of the invention, the process of the invention is carried under conditions likely to promote crosslinking and hydrogel formation (for example high concentrations of reagents with none present in excess) or in the presence of agents such as diamines likely to promote crosslinking. Formation of hydrogels containing modified biological elements would generally be performed using the chemical approaches described in Subr, V., Duncan, R. and Kopecek, J. (1990) Release of macromolecules and daunomycin from hydrophilic gels containing enzymatically degradable bonds, *J. Biomater. Sci. Polymer Edn.*, 1 (4) 261-278.

The number of reactive groups on the polymer is preferably from 0.5 to 10mol%, more preferably from 1 to 6mol%, most preferably from 2 to 5mol%.

The number of linkages between the polymer and the biological element is preferably three or more, more preferably four or more. The number of linkages may be, for example, 12 or 14. The advantage of having a higher number of linkages is that the polymer modified biological element is more stable.

The two or more linkages between the polymer and the biological element are preferably covalent linkages.

It has been found that biological elements modified in accordance with the invention lose their infectivity completely. The infectivity is preferably replaced by coupling a biologically active agent to the polymer. The biologically active agent is optionally coupled to the polymer either before it is

combined with the biological element or after. Preferably it is coupled to the polymer after the polymer has coated the biological element to avoid it interfering with the coupling reaction.

5 The biologically active agent may be incorporated using the same type of reactive groups used to couple the reactive polymer to the biological element, or may be coupled using different chemistry. In the latter situation a heteromultifunctional reactive polymer (for example containing ONp esters and thiol groups) would be used.

10 The biologically active agent is preferably incorporated onto the surface of a polymer-coated biological element to improve targeting, tissue penetration or pharmacokinetics. The biologically active agent is, for example, a growth factor or cytokine, a sugar, a hormone, a lipid, a phospholipid, a fat, an apolipoprotein, a cell adhesion promoter, an enzyme, a toxin, a peptide, a glycoprotein, a serum protein, a vitamin, a mineral, or an antibody recognising
15 receptor for example a growth factor receptor, tissue-specific antigen or tumour-associated antigen. Several biologically active agents may be coupled to each polymer-modified biological element, including mixtures. Alternatively, a multi-purpose agent may be linked as the biologically active agent directly to the polymer-modified biological element to permit subsequent
20 attachment of desired molecules.

An antibody is preferably used as the biologically active agent to re-target coated biological elements to any desirable biological element, for example, receptors, cells, extracellular environments and other proteins. A wide range of different forms of antibody may be used including monoclonal
25 antibodies, polyclonal antibodies, diabodies, chimeric antibodies, humanised antibodies, bi-specific antibodies, camelid antibodies, Fab fragments, Fc fragments.

A suitable tumour specific antigen for use as the biologically active agent is for example a cancer associated antigen, (such as a carcinoembryonic
30 antigen or α -fetoprotein), neuron specific enolase, prostate specific antigen or major histocompatibility complex I or II.

A suitable multi-purpose protein for use as the biologically active agent to act as a generic linker permitting flexibility of application is protein G (this will bind an antibody, allowing surface modification with any IgG class
35 antibody from most species), protein A (which has properties similar to protein

G), avidin (which binds biotin with very high affinity allowing the incorporation of any biotin labelled element on to the surface), streptavidin (which has properties similar to avidin), extravidin (which has properties similar to avidin), wheat germ agglutinin (which binds sugars), hexahistidine (which allows for gentle purification on nickel chelate columns), GST (which allows gentle purification by affinity chromatography).

A suitable growth factor or cytokine for use as the biologically active agent is for example Brain derived neurotrophic factor, Ciliary neurotrophic factor, b-Endothelial growth factor, Epidermal growth factor (EGF), Fibroblast growth factor Acidic (aFGF), Fibroblast growth factor Basic (bFGF), Granulocyte colony-stimulating factor, Granulocyte macrophage colony-stimulating factor, Growth hormone releasing hormone, Hepatocyte growth factor, Insulin like growth factor-I, Insulin like growth factor-II, Interleukin-1a, Interleukin-1b, Interleukin 2, Interleukin 3, Interleukin 4, Interleukin 5, Interleukin 6, Interleukin 7, Interleukin 8, Interleukin 9, Interleukin 10, Interleukin 11, Interleukin 12, Interleukin 13, Keratinocyte growth factor, Leptin, Liver cell growth Factor, Macrophage colony stimulating factor, Macrophage inflammatory protein 1a, Macrophage inflammatory protein 1b, Monocyte chemotactic protein 1, 2-methoxyestradiol, b-nerve growth factor, 2.5s nerve growth factor, 7s nerve growth factor, Neurotrophin-3, Neurotrophin-4, Platelet derived growth factor AA, Platelet derived growth factor AB, Platelet derived growth factor BB, Sex hormone binding globulin, Stem cell factor, Transforming growth factor- β 1, Transforming growth factor- β 3, Tumour necrosis factor α , Tumour necrosis factor β , Vascular endothelial growth factor, and Vascular endothelial growth factor C.

A suitable sugar for use as the biologically active agent for incorporation by amino derivatisation in the form of a monosaccharide, disaccharide or polysaccharide is, for example, D-Galactose, D-Mannose, D-Glucose, L-Glucose, L-Fucose, and Lactose.

A hormone which is suitable for use as the biologically active agent is, for example, Adrenomedullin, Adrenocorticotrophic hormone, Chorionic gonadotrophic hormone, Corticosterone, Estradiol, Estrinol, Follicle stimulating hormone, Gastrin 1, Glucagon, Gonadotrophin, Growth hormone, Hydrocortisone, Insulin, Leptin, Melanocyte stimulating hormone, Melatonin, Oxytocin, Parathyroid hormone, Prolactin, Progesterone, Secretin, Thrombopoietin, Thyrotropin, Thyroid stimulating hormone, and Vasopressin.

A suitable lipid, fat or phospholipid for use as the biologically active agent for targeting the polymer coated biological element or for providing stealth-like properties is, for example, Cholesterol, Glycerol, a Glycolipid, a long chain fatty acid, particularly an unsaturated fatty acid e.g. Oleic acid, Platelet activating factor, Sphingomylin, Phosphatidyl choline, or Phosphatidyl serine.

A suitable cell adhesion promoter for use as the biologically active agent is, for example, Fibronectin, Laminin, Thrombospondin, Vitronectin, Polycations, integrins or oligopeptide sequences binding integrins or tetraspan proteins.

A suitable apolipoprotein for use as the biologically active agent that may also provide stealth-like properties is, for example, a high density lipoprotein or a low density lipoprotein, or a component thereof.

A suitable enzyme for use as the biologically active agent, for example, to promote mobility of the coated biological element through a particular environment is an enzyme capable of degrading the extracellular matrix (for example a gelatinase, e.g. matrix metallo proteases type I to II, or a hyaluronidase), an enzyme capable of degrading nucleic acids (for example Deoxyribonuclease I, Deoxyribonuclease II, Nuclease, Ribonuclease A), an enzyme capable of degrading protein (for example Carboxypeptidase, plasmin Cathepsins, Endoproteinase, Pepsin, Proteinase K, Thrombin, Trypsin, Tissue type plasminogen activator Urokinase type plasminogen activator), an enzyme allowing rapid detection (for example Luciferase, Peroxidase, b-galactosidase), or other useful enzymes (such as Amylase, Endoglycosidase, Endo-b-galactosidase, Galactosidases, Heparinase, HIV reverse transcriptase, b-hydroxybutyrate dehydrogenase, Insulin receptor kinase, Lysozyme, Neuraminidase, Nitric oxide synthase, Protein disulphide isomerase).

A suitable toxin for use as the biologically active agent to bind a receptor or to interact with cell membranes is, for example, Cholera toxin B subunit, Crotoxin B subunit, Dendrotoxin, Ricin B chain.

A suitable peptide for use as the biologically active agent may be provided by for example, transferrin, Green/blue/yellow fluorescent protein, Adrenomedullin, Amyloid peptide, Angiotensin I, Angiotensin II, Arg-Gly-Asp, Atriopeptin, Endothelin, Fibrinopeptide A, Fibrinopeptide B, Galanin, Gastrin, Glutathione, Laminin, Neuropeptide, Anp-Gly-Arg, Peptides

containing integrin binding motifs, Targeting peptides identified using phage libraries, Peptides containing nuclear localisation sequences, Peptides containing mitochondrial homing sequences.

A suitable serum protein for use as the biologically active agent is for example, Albumin, Complement proteins, Transferrin, Fibrinogen, Plasminogen.

A suitable vitamin or mineral for use as a biologically active agent is for example, Vitamin B₁₂, Vitamin B₁₆ or folic acid.

In this way in accordance with the present invention a polymer modified biological element can be synthesised which is targeted to a highly specific set of cells, e.g. tumour cells. At the same time it has been found that the polymer modified biological element in accordance with the present invention is not rendered inactive by neutralising antibodies. This is believed to be because the biological element is shielded by the polymer. The shielding of the biological element by the polymer has been found to have other advantages including increased shelf life and better resistance to low pH. Also it is possible to purify the biological elements using more aggressive technology than that which is feasible with existing biological elements.

Optionally the polymer is coupled to a radioisotope in order to allow the detection of the biological element e.g. in a biological environment.

The bacteria used in the invention includes, for example, a bacteria used in experimental gene therapy (e.g. *salmonella*) a bacteria or baculovirus used as a biological pesticide (e.g. nuclear polydrosis virus NPD, nonocclude virus NV, granulosus virus or *bacillus thuringiensis*), a bacteria strain useful for degrading oil sludges/spills or a genetically modified version thereof (e.g. *enterobacteriaceae*, *anitratum*, *pseudomonas*, *micrococcus*, *comamonas*, *zanthomonas*, *achromobacter* or *vidrio-aeromonas*), a bacterial strain responsible for reducing sulphur to H₂S in oil (e.g. *petrotoga mobilis*, *petrotoga miotherma*, *desulfotomaculum nigrificnas*, *desulphovibrio*) or a bacterial strain capable of oxidising sulphur from oil (e.g. *rhodococcus sp.* Strain ECRD-1).

In principle any known virus may be used in the present invention as the biological element. The virus is preferably a recombinant genetically engineered virus. The recombinant virus optionally contains a transgene. It will be understood that the term "transgene" is used herein to denote a nucleic

- acid which is not native to a virus. For example a transgene could encode a biologically functional protein or peptide, an antisense molecule, or a marker molecule. The virus is either an RNA or DNA virus and is optionally from one of the following families and groups: Adenoviridae; Alfamoviruses:
- 5 Bromoviridae; Alphacryptoviruses: Partitiviridae; Badnaviruses; Betacryptoviruses: Partitiviridae; Bigeminiviruses: Geminiviridae; Birnaviridae; Bromoviruses: Bromoviridae; Bymoviruses: Potyviridae; Bunyaviridae; Caliciviridae; Capillovirus group; Carlavirus group; Carmovirus virus group; Group Caulimovirus; Closterovirus Group; Commelina yellow
 - 10 mottle virus group; Comovirus virus group; Coronaviridae; PM2 phage group; Corbicoviridae; Group Cryptic virus; group Cryptovirus; Cucumovirus virus $\Phi 6$ phage group; Cystoviridae; Cytorhabdoviruses: Rhabdoviridae; Group Carnation ringspot; Dianthovirus virus group; Group Broad bean wilt; Enamoviruse; Fabavirus virus group; Fijiviruses: Reoviridae; Filoviridae;
 - 15 Flaviviridae; Furovirus group; Group Geminivirus; Group Giardivirus; Hepadnaviridae; Herpesviridae; Hordeivirus virus group; Hybrigeminiviruses: Geminivirida; Idaeoviruses; Ilarvirus virus group; Inoviridae; Ipomoviruses: Potyviridae; Iriodoviridae; Leviviridae; Lipothrixviridac; Luteovirus group; Machlomoviruses; Macluraviruses; Marafivirus virus group; Maize chlorotic
 - 20 dwarf virus group; icroviridae; Monogeminiviruses: Geminiviridae; Myoviridae; Nanaviruses; Necrovirus group; Ncpovirus virus group; Nodaviridae; Nucleorhabdoviruses: Rhabdoviridae; Orthomyxoviridae; Oryzaviruses: Reoviridae; Ourmiaviruses; Papovaviridac; Paramyxoviridae; Parsnip yellow fleck virus group; Partitiviridae; Parvoviridae including adeno-
 - 25 associated viruses; Pea enation mosaic virus group; Phycodnaviridae; Phytorecoviruses: Reoviridae; Picornaviridae; Plasmaviridae; Podoviridae; Polydnaviridae; Potexvirus group; Potyvirus; Poxviridae; Reoviridae; Retroviridae; Rhabdoviridae; Group Rhizidiovirus; Rymoviruses: Potyviridae; Satellite RNAs; Satelliviruses; Sequiviruses: Sequiviridae; Sobcmoviruses;
 - 30 Siphoviridae; Sobcmovirus group; SSVI-Type Phages; Tectirividae; Tenuivirus; Tetraviridae; Group Tobamovirus; Group Tobravirus; Togaviridae; Group Tombusvirus; Tospoviruses: Bunyaviridae; Group Torovirus; Totiviridae; Tymoviruses; Group Tymovirus; Plant virus satellites; Umbraviruses; Unassigned potyviruses: Potyviridac; Unassigned
 - 35 rhabdoviruses: Rhabdoviridae; Varicosaviruses; Waikaviruses: Sequiviridae; Ungrouped viruses.

A particularly preferred virus for use in the invention is a retrovirus,

adenovirus, adenoassociated virus, herpesvirus, papovavirus or poxvirus. Adenovirus is especially preferred.

5 A component of a biological element which is suitable for use as the biological element may be provided by, for example, a viral core or a provirus (from e.g. pox viruses). An example of a viral core is an adenovirus core which is preparable by the method disclosed in Russell_W.C., M., K., Skchel, J.J. (1972). "The preparation and properties of adenovirus cores" Journal of General Virology 11, 35-46 and modifications thereto.

10 A phage which is suitable for use as the biological element is for example one from one of the following families: Lambdoid phages, Inovirus, Leviviridae, Styloviridae, Microviridae, Plectrovirus, Plasmaviridae, Corticoviridae, Satellite bacteriophage, Myoviridae, Podoviridae, T-even phages; an example of a particular phage is MV-L3, P1, P2, P22, Φ 29, SP01, T4, T7, MV-L2, PM2, F1, MV-L51, Φ X174, Φ 6, MS2, M13, Q β , tectiviridae
15 (eg. PRD1).

A fungus which is suitable for use as the biological element is for example one from family Basidiomycetes (which make basidiospores, which include classes eg Gasteromycetes, hymenomycetes, urediniomycetes, ustilaginomycetes). A spore which is suitable for use as the biological element
20 is a basidiospore, actinomycetes, arthrobacter, microbacterium, clostridium, Rhodococcus, Thermomonospora or Aspergillus fumigatus.

The process according to the invention is preferably carried out at a pH greater than 7. More preferably the process is carried out at a pH from 7.4 to 8.2, most preferably about pH 8. Generally speaking the process is faster at
25 higher pH values but the rate of hydrolysis of the polymer is faster too. A suitable buffer is generally used in the process; suitable buffers include phosphate, borate, carbonate or HEPES buffers. The temperature of the process is preferably below room temperature, more preferably from 4 to 10°C. The process is preferably carried out with mixing.

30 The compositions according to the invention can be suitable for *in vitro* use or for use in plants or animals. Where the composition is for use in an animal, especially a mammalian animal, the carrier is preferably a pharmaceutically acceptable carrier.

BRIEF DESCRIPTION OF THE DRAWINGS

In the accompanying drawings

Figure 1: Fluorescamine measurement of free amino groups on virus following surface modification with polymer. Reaction of adenovirus with reactive polymers decreases the number of free amino groups on the surface of the virus. Here the virus is reacted with varying amounts of pHPMA-Gly-Gly-ONp and the residual level of amino groups is determined using the fluorescamine assay.

Figure 2. Spectrophotometric determination of reaction between polymers and adenoviruses. Reaction of reactive polymers with adenovirus can be monitored spectrophotometrically, either by production of free 4-nitrophenol or by loss of reactive ester. Here the reaction is monitored by production of free 4-nitrophenol (A404 nm). The lower line represent the reaction with water (unwanted hydrolysis) whereas the top line shows the rate of reaction in the presence of virus, indicating the reaction with virus plus the rate of hydrolysis.

Figure 3. Reaction of polymer with adenovirus leads to modification of fibre protein. This Western blot compares the signal determined for adenovirus fibre protein by Western blotting of viruses treated with increasing amounts of pHPMA-Gly-Gly-ONp. It can be seen that fibre runs normally when low amounts of polymer are used, but higher amounts of polymer leads to a change in mobility and possibly a change in efficiency of detection for the fibre protein. This indicates covalent modification of the fibre by the polymer, and possibly implicates crosslinking.

Figure 4. Reduction in cytotoxicity of adenovirus following surface modification using a multivalent polymer, pHPMA-Gly-Phe-Leu-Gly-ONp. A shows wild type A549 cells, growing healthily. B shows cells treated with wild type Ad5 virus, indicating significant cytopathic effect. C shows cells treated with mono-pEGylated wild type Ad5 virus, indicating that pEGylation does not remove the cytopathic effect. D shows cells treated with pHPMA-Gly-Phe-Leu-Gly-ONp-treated wild type Ad 5, showing complete abolition of the cytopathic effect, with cells growing normally.

Figure 5. A method for measuring anti-adenovirus antibodies by ELISA.

Solutions containing anti-adenovirus antibodies were pre-incubated with wild type Ad 5 virus or polymer-modified Ad 5v virus, at a range of concentrations of virus. Residual antibodies were then determined using an ELISA system with wild type Ad5 as the ligand. It can be seen that many more
 5 antibodies persisted when solutions were pre-depleted with coated virus than when solutions were pre-depleted with wild type virus. This indicates protection of antibody binding by the presence of the polymer coat.

Figure 6. Wild type Ad5 and polymer-coated Ad5 were visualised by Transmission Electron Microscopy (TEM; magnification x 100 000). Samples
 10 were stained with 2% phosphotungstic acid, pH 7.0. Observation by TEM showed the particle size for both Ad5WT and coated virus to be 80nm in diameter; Ad5WT was negatively stained whereas due to the presence of pHPMA, the coated virus was positively stained.

Figure 7. Reduction of infectivity of Ad5-GFP in A549 cells
 15 following reaction with pHPMA-Gly-Gly-ONp. Ad5-GFP was treated with reactive pHPMA-Gly-Gly-ONp as described in the text. A549 cells were exposed to viruses for 48 h and the level of GFP expression was determined using fluorescence. Coating the virus with pHPMA mediated a significant fall in GFP expression, reflecting a major inhibition of virus infectivity.

Figure 8. Reduction of infectivity of Ad5-GFP in human umbilical
 20 vein endothelial (HUVE) cells following reaction with pHPMA-Gly-Gly-ONp. Ad5-GFP was treated with reactive pHPMA-Gly-Gly-ONp as described in the text. HUVE cells were exposed to viruses for 48 h and the level of GFP expression was determined using fluorescence. Coating the virus with pHPMA
 25 mediated a significant fall in GFP expression, reflecting a major inhibition of virus infectivity.

Figure 9. Analysis of restoration of infectivity in A549 cells by
 retargeting polymer-coated viruses with bFGF or VEGF. Ad5 viruses were reacted with pHPMA-Gly-Phe-Leu-Gly-ONp and then retargeted by reaction
 30 with bFGF or VEGF. Viruses were then incubated with A549 cells, and cellular expression of GFP was measured after 48h. It can be seen that bFGF restored the inhibition of infection mediated by polymer-coating, while VEGF did not. This may reflect the presence of bFGF receptors on these cells, and the low level of VEGF receptors.

Figure 10. Analysis of restoration of infectivity in HUVE cells by retargeting polymer-coated viruses with bFGF or VEGF. Ad5 viruses were reacted with pHPMA-Gly-Gly-ONp and then retargeted by reaction with bFGF or VEGF. Viruses were then incubated with HUVE cells, and cellular expression of GFP was measured after 48h. Presence of both bFGF or VEGF restored infectivity to HUVE cells, although VEGF was more effective, probably reflecting the high level of VEGF receptors expressed on these cells.

Figure 11. Evaluation of the effects of neutralising antibodies to inhibit infection using Ad5-GFP or Ad5-GFP modified with pHPMA-Gly-Phe-Leu-Gly-ONp (referred to as EC82 in the drawing). A549 cells were infected with 10^4 particles/cell Ad5-GFP, or pHPMA-modified Ad5-GFP or bFGF-retargeted pHPMA-modified Ad5-GFP in the presence of various dilutions of rabbit anti-Ad5 serum. At 5 days post-infection GFP fluorescence was measured in the cells, and protein concentration determined. Infectivity of coated virus has been restored by addition of bFGF. Neutralisation is reduced by less than 20% with retargeted coated virus compared with a reduction of 99.2% for Ad5-GFP alone.

MORE DETAILED DESCRIPTION OF PREPARATION METHODS AND EXAMPLES

The following examples and descriptions of stages in synthetic routes for preparation of DNA delivery vehicles constructed in accordance with the invention, and components thereof, serve to further illustrate the present invention, and disclose additional important features thereof. They should not, however, be construed in any way as a limitation thereof.

Unless otherwise stated, molecular weight values quoted for polymers are intended to represent weight average values. In the Examples the virus concentrations are given as the number of viral particles per ml. This is much greater than the corresponding number of infectious units.

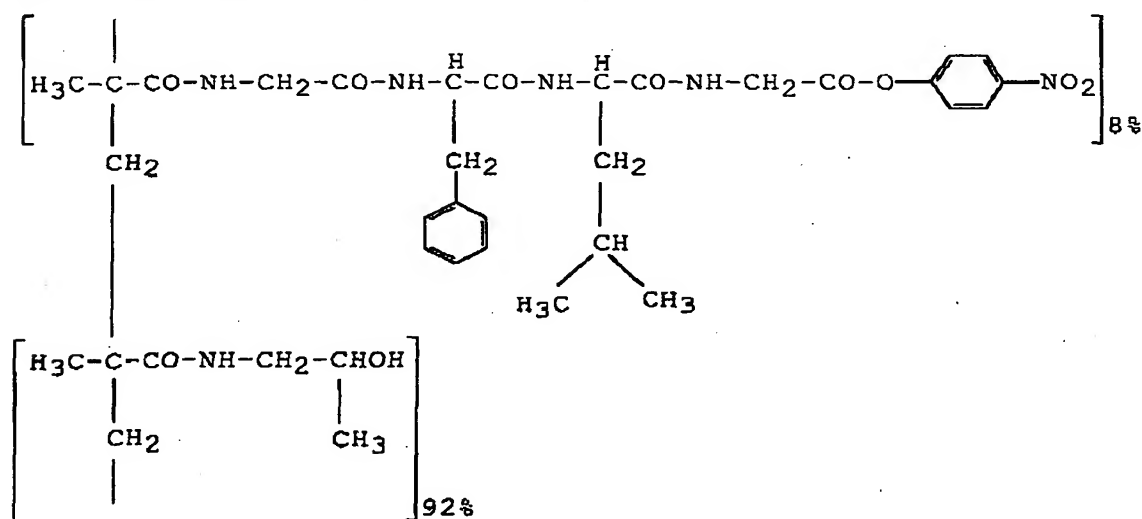
In the first example (EXAMPLE 1), the manner of preparation is described of a adenovirus formed with a protective hydrophilic polymer coating using a preferred 2-step assembly procedure in accordance with the invention.

EXAMPLE 1

Preparation of Nucleic Acid Delivery Vehicle Complex comprising a virus and a Coating of Hydrophilic Polymer formed by Polymeric Precursors based on N-2-hydroxypropylmethacrylamide (HPMA) and reactive esters

This example relates to the formation of a coated virus made up of adenovirus and a so-called polymeric precursor composed of HPMA copolymerised with a methacryloylated-oligopeptide(Glycine-Phenylalanine-Leucine-Glycine) para-nitrophenyl ester.

Such methacrylic polymeric precursors provide the hydrophilic polymer material. They will generally have a molecular weight of about 20,000Da and contain from 4-10 mol% of oligopeptide side chains bearing the activated ester groups (-ONp). The oligopeptide acts as a spacer and may be varied in size, but the tetrapeptide Gly-Phe-Leu-Gly represents one preferred form which is designed for biodegradation by lysosomal cathepsin enzymes (thiol proteinases). A typical structure is shown below:



Preparation of methacrylic polymeric precursors as referred to above generally involves a step of copolymerisation of HPMA with the p-nitrophenyl ester of the N-methacryloylated peptide concerned, and in the "polymeric precursor" so formed the terminal p-nitrophenoxy groups of the peptide side-chains provide convenient leaving groups for subsequent addition reactions with reactive amino or other functional groups of the viruses. The synthesis of p-nitrophenyl esters of N-methacryloylated oligopeptides and their copolymers

with HPMA is well documented in the literature, especially in articles or papers relating to synthetic polymer drug delivery agents, as for example P. Rejmanova *et al* "Aminolyses of Monomeric and Polymeric 4-Nitrophenyl Esters of N-Methacryloylamino Acids", (1977), *Makromol. Chem.* **178**, 2159-2168, Subr. V, *et al*, "Polymers Containing Enzymatically Degradable Bonds", (1992), *Journal of Controlled Release*, **18**, No. 2, pp.123-132 and Ulbrich, K. *et al*, "Polymeric Conjugates of Drugs and Antibodies for Site-Specific Drug-Delivery" *Macromolecular Symposia*, (1996), **103**, pp. 177-192. See also EP-A-0187547 of which the content is incorporated herein by reference.

10 The preparation of hydrophilic poly(HPMA) polymers having side chains bearing reactive p-nitrophenyl esters or reactive p-nitrophenoxy groups is more particularly described in Examples 3 and 4 of WO 98/19710.

It is incidentally also possible for constructing the hydrophilic polymer to use polymeric precursors of HPMA copolymerised with N-methacryloylated oligopeptides in which the peptide side chains terminate in carboxyl groups instead of p-nitrophenyl ester groups, prepared as described in EP-A-0187547. In the presence of suitable catalysts (again see EP-A-0187547) the carboxyl groups will bind to primary amino groups on the viruses, as with nitrophenyl esters. However, an additional possibility also arises in that the carboxyl groups may bind to reactive alcohol groups in the virus, forming ester groups. In that event such ester groups may be subsequently broken down through acid-catalysed or hydrolytic degradation.

Adenovirus (25 µg, equivalent to 8.6×10^{10} viral particles) was incubated in 100 µl phosphate buffered saline(PBS)/glycerol (10 % vol/vol) containing 50 mM HEPES buffer pH 7.4 at 6°C. The hydrophilic polymer (polymeric precursor) bearing reactive ester groups was dissolved in water and added to the mixture with gentle mixing (final concentration 2.5 mg/ml). It is important that the solution does not contain nucleophilic groups such as reactive amines (eg. Tris buffer) which could react with the ester groups. It is also important that the solution should not become too alkaline (eg. pH not > 8.2) as this will promote unwanted hydrolysis of the activated ester groups. It is also important that the pH of the virus solution does not fall below 7.0 as this will begin to inactivate the adenovirus.

The reaction between the -ONp ester groups and the primary amino functions of the virus was monitored spectrophotometrically, either by measuring decreasing concentration of the esters (274 nm) or by monitoring

appearance of free 4-nitrophenol (404 nm). After 1 h aminoethanol (0.1 %, vol/vol) was added to terminate the reaction. In some instances, for application of viruses at high concentrations, free 4-nitrophenol is subsequently removed by filtration or dialysis before application to cells.

- 5 Optimal reaction conditions include gentle mixing (but not vortexing) either in HEPES buffer, borate solution or in water at pH 7.0 – 8.2, temperature of 4 – 10 °C and a maximal virus concentration of 10^{13} particles/ml to minimise crosslinking.

- 10 In some instances the pH may be gradually raised during the reaction, either by addition of sodium hydroxide or a higher pH buffer, up to about pH 8.0. This promotes reactivity of the primary amino groups, but must be regulated carefully as it also accelerates the rate of ester hydrolysis.

- 15 The reaction may be terminated either by raising the pH to 8.5, promoting rapid ester hydrolysis, or preferably by addition of low molecular weight reactive amines, e.g. aminoethanol, aminopropanol or ethylenediamine.

It has also been found that the coated viruses are relatively stable and easy to handle, and they can be purified by column chromatography (eg. Sepharose 4B-CL) or by density gradient centrifugation.

- 20 Synthesis of the reactive hydrophilic polymer used in the above example which contains tetrapeptide-paranitrophenyl esters, has already been referred to. Careful selection of the reactive hydrophilic coating polymer can significantly affect the properties of the resulting coated viruses. For example, use of polymers having simple oligopeptide-nitrophenol ester reactive side chains leads to aminolytic reaction with uncharged amino groups of the viruses
25 with release of p-nitrophenol, but there is also a significant component of hydrolysis. The hydrolytic product is a free carboxylic acid at the terminal amino acid, and hence such coated viruses are often found to possess strongly negative surface charges (eg. zeta potential of -25 mV). Alternative chemistry, for example using carbonate esters of paranitrophenol yield the same products
30 on aminolysis, with release of carbon dioxide, but produce hydroxyl groups following hydrolysis. The measured zeta potential of the resulting coated viruses is generally very close to zero.

- 35 Several other reactive hydrophilic polymers can be used to achieve surface-coating of viruses. These include reactive esters based on other polymer backbones, such as poly-N5-(2-hydroxyethyl)-L-glutamine (pHEG), or

reactive polymers containing backbones composed primarily of blocks of poly(ethylene glycol) joined end-to-end by oligopeptide or other biodegradable sequences bearing pendant reactive esters. Careful selection of the structure of these molecules can tailor them for degradation by specific enzymes, in specific locations, or for hydrolytic or acid-catalysed hydrolytic degradation. The synthesis of some of these materials is described in later examples, and they make particularly effective agents for surface modification of viruses using the same protocol as described above. Reactive hydrophilic polymer material based on poly-N-(2-hydroxyethyl-L-glutamine) (pHEG), containing reactive ONp carbonate esters with no amino acid spacer, can be produced by reaction of pHEG with chloroformate and is known to be readily biodegradable.

Coated viruses formed with a net strong negative surface charge are subject to rapid scavenging by phagocytic cells, notably Kupffer cells, following intravenous administration. Coated viruses bearing net positive charges are prone to accumulation in capillary beds, notably the pulmonary capillaries.

Accordingly, the best surface charge for achieving prolonged plasma circulation is neutral or slightly negative.

As will be appreciated, other bioactive molecules, such as targeting groups or additional shielding molecules, may be attached to the hydrophilic polymer precursor. In an example described below (EXAMPLE 2), the targeting agent transferrin has been incorporated by simple aminolysis or following oxidation of its carbohydrate component.

EXAMPLE 2

25 Construction of transferrin-targeted coated viruses using aminolysis

E1-disabled adenovirus (25 μ g, equivalent to 8.6×10^{10} viral particles) encoding the β -galactosidase reporter gene under the control of the cytomegalovirus immediate-early promoter in E1 was incubated in 100 μ l phosphate buffered saline(PBS)/glycerol (10 % vol/vol) containing 50 mM HEPES buffer pH 7.4 at 6°C. Reactive hydrophilic polymer (pHPMA bearing 8 mol % Gly-Phe-Leu-Gly-4-nitrophenol esters, as from Example 1) was added (final concentration 2.5 mg/ml). The reaction was allowed to proceed for 1 h before addition of holotransferrin (20 μ g) and the reaction was then allowed to continue for a further 1 h. At that time the reaction was ended by addition of

aminoethanol (0.1 % vol/vol), viruses were allowed to stand for a further 30 min and then purified by dialysis.

As an alternative the above reaction may be performed in an automatic titration apparatus (e.g. a pH-Stat from Radiometer) maintained at 6 °C and
5 programmed to maintain the pH at 7.4

Coated viruses were then incubated in medium containing 2 % foetal calf serum with transferrin receptor-positive K562 cells (10^4 cells/well in 96-well plates), final virus concentration 10^8 particles/well. After 72 h cells were lysed in phosphate buffer (100 mM, pH 7.2 containing Triton X-100 (0.1 %, vol/vol)) prior to measurement of β -galactosidase reporter gene expression
10 using a commercial Galactolight™ luminescence assay kit. Transferrin-targeted coated viruses were found to mediate significantly higher gene expression than non-targeted polymer-coated viruses, and this transfection activity could be inhibited by the addition of excess competing free transferrin.
15 When incubated with transferrin receptor-negative 293 cells, however, these transferrin-targeted complexes gave no transfection activity.

Despite this demonstration of transferrin-targeted gene expression using coated viruses, the result was rather unexpected since it was previously unclear whether the coated viruses would be able to leave the
20 endosome/lysosome system and enter the cytoplasm. This example demonstrates the ability of coated viruses to function effectively following their entry into cells in transferrin-mediated internalisation.

EXAMPLE 3

**Construction of transferrin targeted coated viruses using carbohydrate
25 oxidation**

Adenoviruses were coated with a reactive hydrophilic polymer as described above. In contrast to Example 2, however, no transferrin was used and the coating reaction was terminated by the addition of a 20-fold molar excess of diaminoethylene. This resulted in the incorporation of amino groups
30 onto the surface of the coated viruses via the remaining unreacted ONp ester groups. The amino group-bearing coated viruses were purified from free diamine and polymer by gel filtration on Sepharose 4B-CL with distilled water as eluent.

For the oxidation of the transferrin carbohydrate chain, 10 mg transferrin
35 (0.13 μ mol) was dissolved in 0.45 ml of sodium acetate buffer (pH 5.0, 30 mM)

and chilled to 0°C. Freshly dissolved sodium periodate (50 µl of a 10 mg/ml solution) was added and the reaction was performed for 90 min at 0°C in the dark. The oxidised transferrin was purified by gel filtration on prepacked PD10 columns (Pharmacia) and the presence of aldehyde groups was demonstrated using the anisaldehyde test. The oxidised transferrin was kept at pH 5.0 to prevent autoreaction.

For linkage of the oxidised transferrin to the amino function-bearing coated viruses the pH was adjusted to 7.4 and an appropriate amount of oxidised transferrin was added to purified coated complexes. The mixture was left for 1-2 hrs to permit formation of Schiff's base type covalent linkages. The Schiff's bases were subsequently stabilised by reduction for a minimum of 1 hr using an excess of cyanoborohydride. Finally the viruses were purified from unincorporated transferrin and cyanoborohydride and sterilised by gel filtration on Sepharose 4B-CL or equivalent with PBS pH 7.4 as eluent.

Biological activity was demonstrated as described above.

EXAMPLE 4

Construction of transferrin targeted polymer-coated adenoviruses using a heterobifunctional crosslinker

This procedure involved production of polymer-coated viruses bearing SH groups and their conjugation with SH-reactive transferrin molecules.

Viruses were prepared as described above. Cysteamine (2-aminoethane thiol) was reacted with the pHPMA-based reactive coating polymer at different ratios (from 2 to 25 % equivalent to the reactive esters) prior to addition to the viruses. This reaction was carried out in a pH-Stat (Radiometer) at pH 7.4 and 16°C, as follows:

An appropriate amount of the precursor form of the hydrophilic coating material (for example 400 µg/ml of a pHPMA-based copolymer with 8 mol% activated ester group) was dissolved in water and the desired amount of cysteamine was added. The reaction of the polymer precursor with cysteamine was started by raising the pH to 7.4. The reaction is very rapid and is essentially complete after 3 min. The modified polymer precursor was stored at pH 6.0 to prevent unwanted hydrolysis.

An equal volume of adenovirus was then added to the modified polymer precursor. The reaction of the coating material with the amino groups of the virus was initiated by increasing the pH to 7.4, and allowed to proceed for 2 hrs. Unreacted ONp ester groups were then reacted with an excess of aminopropanol.

Sulphide-reactive transferrin was prepared as follows: Transferrin was dissolved in water at 25 ng/ml. Between 1 - 2 molar-equivalents of succinimidopyridyldithiopropionate (SPDP) was added (for 1 ml of 25 mg/ml transferrin, around 20 μ l of a 10 mg/ml SPDP solution would be used). The mixture was left for 1 hr at room temperature before being subject to gel filtration using a PD10 column.

The sulphide-reactive pyridyldithiopropionate-transferrin (PDP-Tf) was now reacted onto sulphide-bearing coated viruses. This was achieved by adding an appropriate amount of the PDP-Tf to a solution of coated viruses prepared as described above under neutral conditions. The exchange reaction was allowed to proceed overnight.

All reactions were carried out in an oxygen-free atmosphere using degassed solutions to prevent formation of disulphide bonds by the coating polymer, potentially leading to the formation of aggregates. For the same reason a molar excess of sulphide-reactive transferrin over sulphide groups of the coating material was used. After completion of all reactions, the viruses were purified from non-incorporated materials and reaction by-products and sterilised by aseptic gel filtration on Sepharose 4B-CL or other suitable matrices. Phosphate buffered saline was used as the eluent. Biological activity was demonstrated as described above.

EXAMPLE 5

Reaction of the Virus complex with pEG-SH

Adenoviruses were incubated and surface-modified using a multivalent polymer bearing reactive esters and free thiol groups as described in Example 4. The coated virus was mixed under argon with a solution of 100mg pEG-SH in 3ml phosphate buffer, pH 7.4 (oxygen-free). The reaction was carried out at room temperature for 4 hours. The pEG-containing complex produced was examined by agarose gel electrophoresis, atomic force microscopy, and photon correlation spectroscopy. The grafting of pEG (5 mol%) via disulfide bonds was confirmed by UV spectroscopy (absorption at 412nm) after reaction with

DTT, followed by quantitative determination of the pEG-SH released using 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB).

EXAMPLE 6

- 5 **Coating of viruses with reactive hydrophilic polymers which are biodegradable in the polymer backbone.**

In this example viruses were stabilised by surface modification using a hydrophilic polymer bearing pendant reactive esters. The hydrophilic polymer used was formed from alternating blocks of poly(ethylene glycol) and
10 tripeptides, designed to introduce proteolytic degradability into the polymer backbone and was prepared as described in parts 11.1 and 11.2 of Example 11 of WO 98/19710.

Stabilisation of viruses by surface modification using pEG-peptide-ONp repeating polymer.

- 15 Reactive pEG-peptide-ONp repeating polymer (dissolved in DMSO) was added to viruses in PBS/glycerol (10%) containing HEPES buffer (50mM) (pH 7.4) to a final concentration of 200 µg/ml. The solution went gradually yellow as free paranitrophenol was released. Infectivity of viruses was determined by titring against A549 cells, and was decreased by the presence of
20 the polymer coating.

EXAMPLE 7

Surface modification of biological elements bearing aldehyde groups using polymers derivatised with hydrazide groups.

- 25 Attachment of surface coating polymers through bonds which are acid unstable and labile at endosomal pH is one important aspect of this invention. There are several chemical strategies suitable for this purpose, but one is exemplified here.

Synthesis of hydrazide-modified hydrophilic polymer

- 30 (a) 1st stage: Coupling of pHPMA-ONp with succinic acid t-butyl-oxycarbonylhydrazide

0.2 g pHPMA-ONp was aminolysed with ethylene diamine at pH 8.0 in HEPES buffer. The material was dialysed into water and mixed with

succinic acid t-butyloxycarbonylhydrazide (20.64 mg, 0.156 mmol). The pH of the solution was adjusted to 5.0 with HCl. Subsequently, 300 mg of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (1.56 mmol) in water was added to this solution. The pH was maintained at 5.0 with HCl during the reaction. After stirring overnight, the solution was dialysed for 48 h against water. The polymer was collected by freeze drying. The degree of substitution was determined by means of $^1\text{H-NMR}$ and was 4%.

(b) 2nd stage: Removal of the t-butyloxycarbonyl protecting group

0.2 g of this modified pHPMA derivative (above) was dissolved in 10 ml trifluoroacetic acid. The mixture was stirred for 1 h and the solvent was evaporated. The residue was dissolved in water and further dialysed for 48 h. The polymer was collected by freeze-drying. The degree of substitution was determined by means of $^1\text{H-NMR}$ and was found to be 3.8 %.

(c) 3rd stage: Grafting of *Aspergillus fumigatus* spores with the hydrazide-modified pHPMA.

Spores of *Aspergillus* (50 μg) were incubated hydrazide-modified pHPMA (200 μg) in citrate buffer at pH 5.0 and stirred for 2 h. The amount of pHPMA grafted onto the spores was calculated following determination of spore-associated aldehyde groups using Benedict's reagent.

EXAMPLE 8

Determination of pHPMA-Gly-Gly-ONp modification of wild type adenovirus type 5 (wt Ad5) particles by fluorescamine assay.

To quantify the extent of amino group modification of wt Ad5 surface proteins, pHPMA-Gly-Gly-ONp treated adenovirus was compared to unmodified Ad5 using amino reactive fluorescamine.

25 μg (8.6×10^{10}) of wt Ad5 particles in 100 μl of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0-250 μg of pHPMA-Gly-Gly-ONp for 1 hour on ice. 1mg of this preparation was diluted in 375 μl of PBS glycerol (10%) and made up to 500 μl with acetone containing 100 $\mu\text{g}/\text{ml}$ fluorescamine. After 5 minutes reaction with fluorescamine, fluorescence was measured at 392ex:480em in a quartz cuvette. Results were expressed as percentage of amino groups lost compared with untreated virus, and the

background signal for un-reacted fluorescamine on its own was removed (Figure 1).

EXAMPLE 9

5 **Measurement of pHPMA-Gly-Phe-Leu-Gly-ONp reaction with wt Ad5 by release of 4-nitrophenol.**

The reaction of pHPMA with amino groups or by hydrolysis yields free 4-nitrophenol which can be measured at 404 nm. Alternatively loss of the reactive ester can be monitored at 274 nm.

10 100µg of wt Ad5 in 1000µl PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 500µg of pHPMA-Gly-Phe-Leu-Gly-ONp, and production of free paranitrophenol (OD 404 nm) was measured in a 1ml quartz cuvette measured as a function of time. The rate of reaction was compared to pHPMA-Gly-Phe-Leu-Gly-ONp without virus to get a background level of hydrolysis (Figure 2) and the presence of the virus stimulated significantly
15 faster reaction, indicating the formation of covalent bonds between the virus and the polymer.

EXAMPLE 10

Demonstration of wt Ad5 fiber modification by pHPMA-Gly-Gly-ONp by western blot.

20 To determine changes in mobility imposed on viral proteins following reaction with pHPMA-Gly-Gly-ONp, coated viruses were analysed by SDS-PAGE and transferred to nitrocellulose paper for detection with antibodies (in this case anti-fiber antibody).

25 25µg (8.6×10^{10}) of wt Ad5 particles in 100µl of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0 - 250µg of pHPMA-Gly-Gly-ONp for 1 hour on ice. Subsequently, 0.1% amino ethanol was added to complete the reaction with any spare ester groups. 2µg (6.9×10^9) wt Ad5 particles were denatured and separated on a 12% SDS PAGE gel for 2 hours at 200V. Proteins were then transferred to nitrocellulose and electrophoresed for
30 45 minutes at 10V. The nitrocellulose membrane was then blocked with 5% Marvel in 0.1% Tween PBS for 1 hour at room temperature before being probed with 1/1000 dilution of guinea pig anti Ad5 fiber antibody in 0.1% Tween PBS for 1 hour. The membrane was washed 3 times for 10 minutes in

0.1% Tween PBS and exposed to 1/10000 dilution of anti guinea pig peroxidase conjugate (SIGMA) in 0.1% PBS Tween for 1 hour. (Figure 3).

EXAMPLE 11

5 Reduction of cytopathic effect of wt Ad5 by addition of pHPMA-Gly-Gly-ONp

wt Ad5 will infect many cell types with high efficiency, this event causes an obvious change in cell morphology called a cytopathic effect (CPE). Modification of wt Ad5 with a multivalent hydrophilic polymer, in this case
 10 pHPMA-Gly-Gly-ONp, reduces its ability to produce a cytopathic effect as observed by the following protocol.

25µg (8.6×10^{10}) of wt Ad5 particles in 100µl of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0-250µg of pHPMA-Gly-Gly-ONp for 1 hour on ice. Subsequently, 0.1% amino ethanol was added to
 15 complete the reaction with any spare ester groups. 0.25µg (8.6×10^8) of this preparation was diluted in 500µl of DMEM 10% foetal calf serum, supplemented with 2 mM glutamine and incubated with a monolayer of A549 cells (10^5) in a 24 well plate. Images of the cell monolayers were then captured with a digital camera after 48 hours (figure 4). Note, 8.6×10^8 pHPMA-Gly-
 20 Gly-ONp treated adenovirus particles were unable to cause a CPE this is 860 times the value that is normally required for adenovirus to produce a 100 % CPE.

EXAMPLE 12

25 Reduction of antibody interaction with pHPMA-Gly-Phe-Leu-Gly-ONp treated adenovirus.

The ability of adenovirus to infect target cells can be compromised by the presence of neutralising antibodies. In this preparation, which is unlikely to be optimum, a substantial loss in antibody binding is observed.

30 20ng of heat treated wt Ad5 (56°C for 30mins) was added in 100µl to wells of a 96 well plate for 2 hours in PBS glycerol (10%). Meanwhile, serial two fold dilutions of wt Ad5 or pHPMA-Gly-Phe-Leu-Gly-ONp treated wt Ad5 (maximum concentration 5µg/ml) were pre-incubated with rabbit anti Ad5

serum (1/1000) in PBS 0.1% Tween for 1 hour. The pre-incubated mixture was then added to the washed 96-well plate (3x PBS 0.1% tween, 3x PBS) coated with wt Ad5 for two hours to bind any remaining antibodies. The plate was then washed again (3x PBS 0.1% tween, 3x PBS) and a secondary anti rabbit
 5 peroxidase conjugate (1/10000) was added in PBS 0.1% tween for a further 2 hours. The colour reaction was initiated by addition of OPD reagent dissolved in 0.1M citrate buffer for 30 minutes, the reaction was then stopped with 50µl of 4M H₂SO₄ and read at 490nm (figure 5).

10 EXAMPLE 13

Loss of Ad5-GFP ability to express reporter gene in A549 cells after treatment with pHPMA-Gly-Gly-ONp and characterisation of coated viruses by electron microscopy.

Coating adenovirus with a multifunctional hydrophilic polymer, in this
 15 case pHPMA-Gly-Gly-ONp, prevents gene expression as recorded by the green fluorescence protein reporter gene.

25µg (8.6×10^{10}) of Ad5-GFP particles in 100µl of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0-250µg of pHPMA-Gly-Gly-ONp for 1 hour on ice. Subsequently 0.1% amino ethanol was added to
 20 complete the reaction with any spare ester groups. Coated and non-coated viruses were visualised using transmission electron microscopy. Changes in affinity for the phosphotungstic acid between uncoated and coated viruses indicated the presence of a polymer surface coat, and coated viruses showed a very slightly larger diameter, consistent with the presence of a surface coating
 25 of the polymer. There was also an indication of a small amount of aggregation following polymer-coating, with some viruses appearing as dimers or trimers (Figure 6). 10^7 - 10^9 particles of this preparation was added to 10^4 A549 cells in a 96 well plate in DMEM containing 2% foetal calf serum, supplement with 2mM Glutamine. After 72 hours cells were lysed using 100mM potassium
 30 phosphate buffer with 0.2% Triton pH 7.2 and fluorescence was determined using a 96-well fluorimeter. (figure 7) demonstrates complete loss of activity after the coating procedure.

EXAMPLE 14**Loss of Ad5-GFP ability to express reporter gene in HUVE cells after treatment with pHPMA-Gly-Gly-ONp**

Coating adenovirus with a multifunctional hydrophilic polymer, in this case pHPMA-Gly-Gly-ONp, prevents gene expression as recorded by the green fluorescence protein reporter gene.

25µg (8.6×10^{10}) of Ad5-GFP particles in 100µl of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0-250µg of pHPMA-Gly-Gly-ONp for 1 hour on ice. After which, 0.1% amino ethanol was added to complete the reaction with any spare ester groups 10^7 - 10^9 particles of this preparation was added to 10^4 HUVE cells in a 96 well plate in DMEM containing 2% foetal calf serum, supplemented with 2mM Glutamine. After 72 hours cells were lysed using 100mM potassium phosphate buffer with 0.2% triton pH 7.2 and fluorescence was determined using a 96-well fluorimeter. (figure 8) demonstrates complete loss of activity after the coating procedure.

EXAMPLE 15**Analysis of restoration of gene expression in HUVE and A549 cells following retargeting of pHPMA-Gly-Gly-ONp – modified viruses with bFGF or VEGF**

Coating adenovirus with a multifunctional hydrophilic polymer, in this case pHPMA-Gly-Gly-ONp prevents gene expression as recorded by the green fluorescence protein reporter gene (Examples 13 and 14).

25µg (8.6×10^{10}) of Ad5-GFP particles in 100µl of PBS/glycerol (10%) and 50mM HEPES pH 7.4 were treated with 250µg of pHPMA-Gly-Phe-Leu-Gly-ONp for 1 hour on ice. 10µg of bFGF or VEGF was then added for a further 1 hour. Subsequently, 0.1% amino ethanol was added to complete the reaction with any spare ester groups. 10^7 - 10^9 particles of this preparation was added to 10^4 A549 cells in each well of a 96 well plate in DMEM containing 2% foetal calf serum, supplemented with 2mM Glutamine. After 72 hours cells were lysed using 100mM potassium phosphate buffer with 0.2% triton pH 7.2 and fluorescence was determined using a 96-well fluorimeter. Figure 9 demonstrates that the complete loss of activity observed following application of the coating procedure can be reversed by incorporation of bFGF as targeting agent. bFGF-retargeted polymer-coated adenovirus shows levels of gene expression comparable with the parental Ad5-GFP. In contrast, VEGF-

retargeted coated virus shows little activity, probably reflecting the low number of VEGF receptors present on these cells.

The same procedure was applied to HUVE cells, except Ad5-GFP was modified with pHPMA-Gly-Gly-ONp. Figure 10 shows that the abolition of infectivity measured using HUVE cells following polymer-coating of Ad5-GFP can also be reversed by incorporation of bFGF as targeting ligand. In these cells, retargeting with bFGF yields infectivity comparable with that observed using the parental non-coated virus. VEGF also acts as an effective retargeting agent in these cells, consistent with the high level of VEGF receptors expressed on HUVE cells, and mediates levels of infectivity greater than those achieved using the non-coated virus.

EXAMPLE 16

Synthesis of amino-terminated poly(HPMA)

Amino-terminated poly(HPMA) polymers were prepared by radical polymerisation of HPMA in the presence of 2-aminoethanethiol.hydrochloride (AET) as the chain transfer agent. The polymers were obtained by 24 h polymerisation at 50 °C in methanol. Concentration of the reagents in polymerisation mixture were as follows: HPMA 0.79 M, initiator AIBN 3.3×10^{-3} M, AET 5×10^{-3} – 5×10^{-2} M. The polymers with molecular weights in the range of 2000 to 20000 g/mol were isolated by precipitation into a 20-fold excess of acetone – diethylether (3:1). The molecular weights of the polymers were determined by FPLC on Superose 12 column in 0.05 M TRIS buffer pH 8.0 containing 0.5 M NaCl as a mobile phase. The content of the terminal amino groups was determined by a colorimetric assay using 2,4,6-trinitrobenzenesulfonic acid.

EXAMPLE 17

Synthesis of poly[(HPMA)-co-(Methacyloyl (MA)-Gly-Phe-Leu-Gly-ONp)]-graft-poly(HPMA)

Poly[(HPMA)-co-(MAGly-Phe-Leu-Gly-ONp)] was dissolved in dry DMSO and calculated amount of amino-terminated poly(HPMA) was added to modify the required part of active ester groups. The solution was stirred for 12 hours at 25 C. The polymer was isolated by precipitation into a 20-fold excess

of acetone – diethylether (3:1 by vol) and reprecipitated from 20 % methanol solution into acetone.

The residual content of *p*-nitrophenyl active ester groups was determined by measuring UV absorption at 280 nm in DMSO. The obtained polymer was characterised (both number and weight average molecular weights) by FPLC equipped with on-line 18-angle light scattering detector WYATT.

EXAMPLE 18

Synthesis of poly[(HPMA)-*co*-(MAGly-Phe-Leu-GlyONp)]-graft-oleylamine

Poly[(HPMA)-*co*-(MAGly-Phe-Leu-Gly-ONp)] was dissolved in dry DMSO and calculated amount of oleylamine was added to modify the required part of active ester groups. The solution was stirred for 2 hours at 25 C. The polymer was isolated by precipitation into 20-fold excess of acetone – diethylether (3:1 by vol) and reprecipitated from 20 % methanol solution into acetone.

The residual content of *p*-nitrophenyl active ester groups was determined by measuring UV absorption at 280 nm in DMSO. The content of oleyl groups was estimated by ¹H-NMR in DMSO-d₆ using the signal of oleyl double bond (5.3 ppm). The obtained polymer was characterised (both number and weight average molecular weights) by FPLC equipped with on-line 18-angle light scattering detector produced by WYATT.

EXAMPLE 19

Surface modification of Vaccinia virus with pHPMA-GLY-GLY-ONp reduces infectivity by plaque assay

Vaccinia virus was selected as a representative member of the Pox virus family, to demonstrate surface modification using pHPMA-based multivalent polymers. Surface modification of Vaccinia virus with a multifunctional hydrophilic polymer can be measured by monitoring a reduction in natural infection activity. Infection activity can be subsequently restored to within one order of magnitude of wild type levels by incorporation of bFGF using the following protocol: note, it was found that low concentrations (< 20µg/ml) and brief sonication was necessary to reduce crosslinking of virus particles.

- 10µg ($\sim 2.3 \times 10^9$) particles of Vaccinia virus in 100µl of PBS and 50mM HEPES pH 7.4 were treated with 100µg of pHPMA-GLY-GLY-ONp for 1 hour on ice. 10µg of bFGF was then added for a further 1 hour. Subsequently, 0.1% (vol/vol) amino ethanol was added to complete the reaction with any spare ester groups. Virus titer was measured by serial dilution of particles on 20mm plates containing 75% confluent HcLa cells. Plaques were counted after 72h with the aid of crystal violet staining.

EXAMPLE 20

10 Surface modification of retrovirus using pHPMA-Gly-Gly-ONp

- To demonstrate surface modification of RNA containing enveloped viruses, the retrovirus rv.AM12.LNC expressing nitroreductase (NTR) gene was selected. NTR expression increases the sensitivity of the cell line SKOV3 to a prodrug (CB1954) by converting it to the active bi-functional alkylating species; this resulting toxicity was used as a measure of infectivity. Surface modification of rv.AM12.LNC with pHPMA-GLY-GLY-ONp reduced NTR expression levels to below detectable levels. However, expression of NTR and sensitisation of SKOV3 cells to CB1954 was restored to within 50% of wild type levels after incorporation of bFGF using the following protocol.

- 20 Concentrated retrovirus particles, (5×10^6 plaque forming units PFU) in 100µl of PBS and 50mM HEPES pH 7.4 were treated with 100µg of pHPMA-GLY-GLY-ONp for 1 hour on ice. For retargeting modified viruses, 10µg of bFGF was then added for a further 1 hour. After which, 0.1% (vol/vol) amino ethanol was added to complete the reaction with any spare ester groups. 500ml of this preparation was diluted to 5mls in DMEM containing 10% foetal calf serum supplemented with 2mM glutamine and added to 10^6 SKOV3 cells in a 10cm plate in the presence of polybrene.

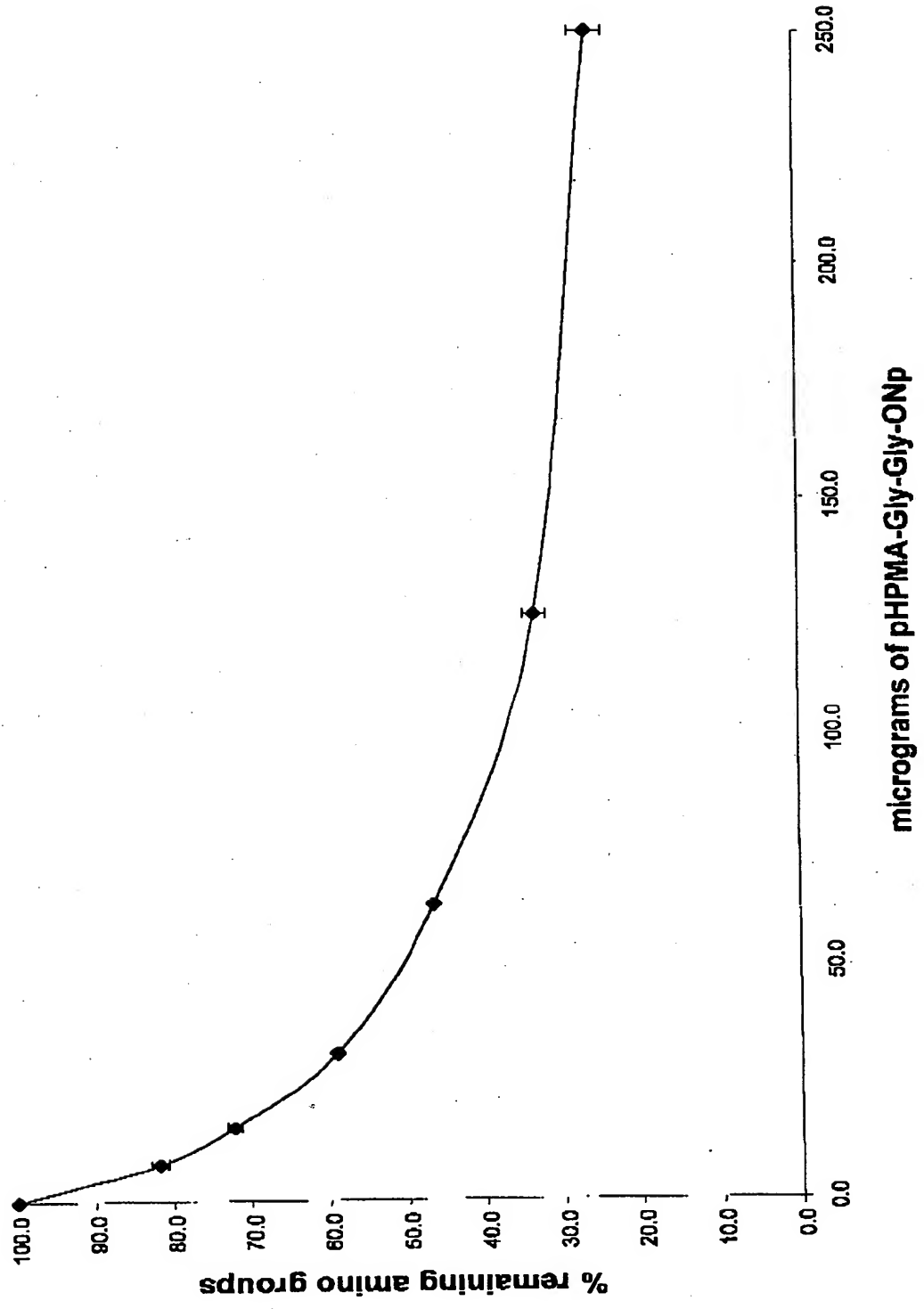
EXAMPLE 21

30 Resistance to inhibition by neutralising antibodies of transfection activity of polymer-coated adenoviruses retargeted using bFGF in A549 cells

Coating adenovirus with a multifunctional hydrophilic polymer, in this case pHPMA-Gly-Gly-ONp prevents gene expression as recorded by the green fluorescence protein reporter gene.

25 μ g (8.6×10^{10}) of Ad5-GFP particles in 100 μ l of PBS glycerol (10%) and 50mM HEPES pH 7.4 were treated with 0-250 μ g of pHPMA-Gly-Gly-ONp for 1 hour on ice. 10 μ g of bFGF was then added for a further 1 hour. Subsequently, 0.1% amino ethanol was added to complete the reaction with any spare ester groups. 10^7 - 10^9 particles of this preparation were pre-incubated in rabbit anti Ad5 hyperimmune serum for 30 minutes before addition to 10^4 HUVE cells in each well of a 96 well plate in DMEM containing 2% foetal calf serum, supplemented with 2mM Glutamine. After 72 hours cells were lysed using 100mM potassium phosphate buffer with 0.2% triton pH 7.2 and fluorescence was determined using a 96-well fluorimeter. (figure 11) demonstrates complete loss of activity after the coating procedure.

Figure 1.



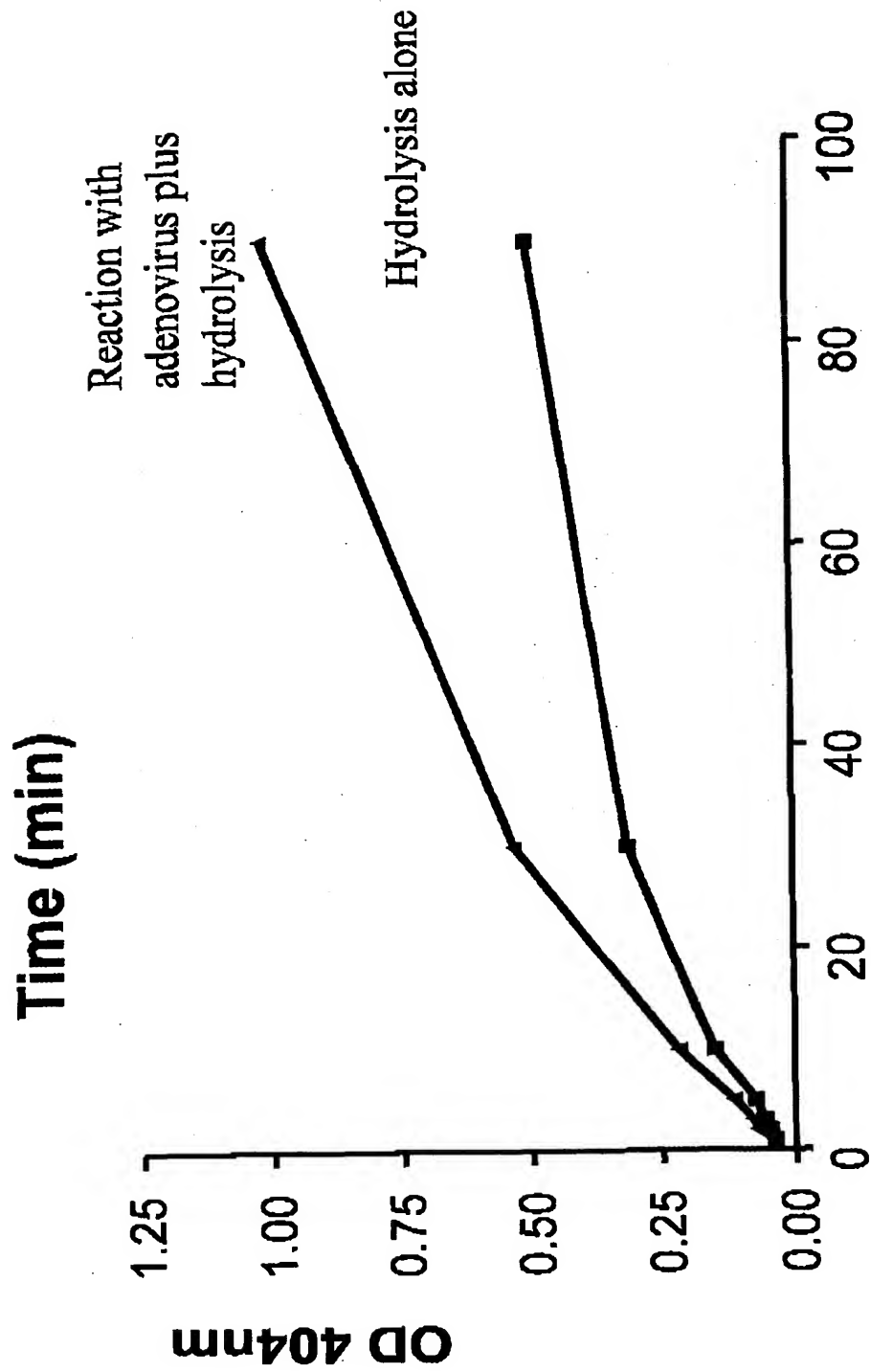


Figure 3.

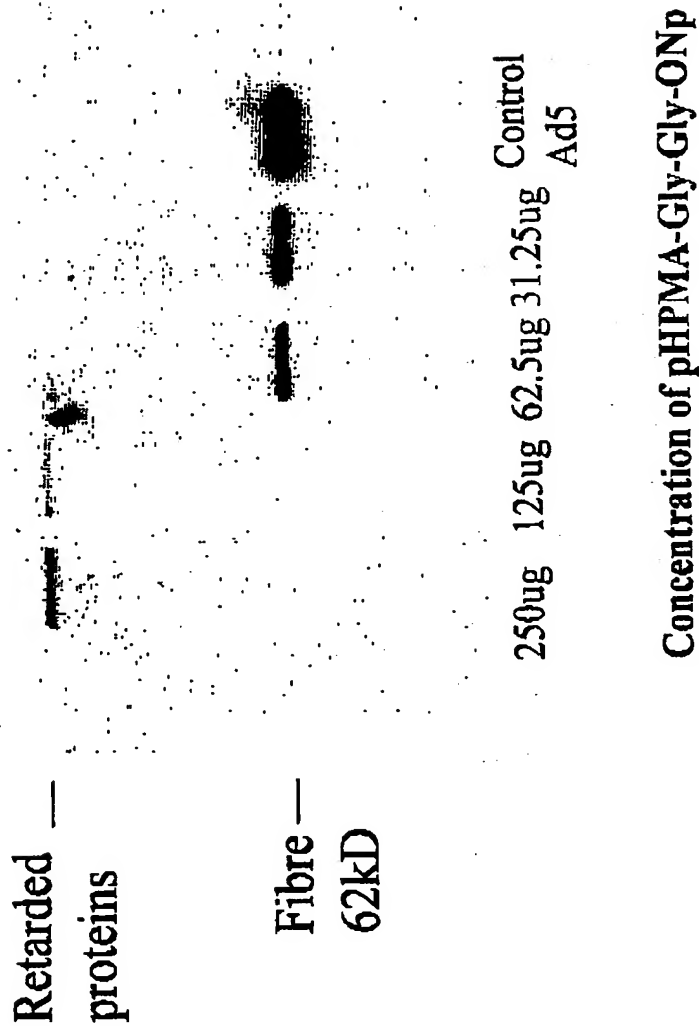
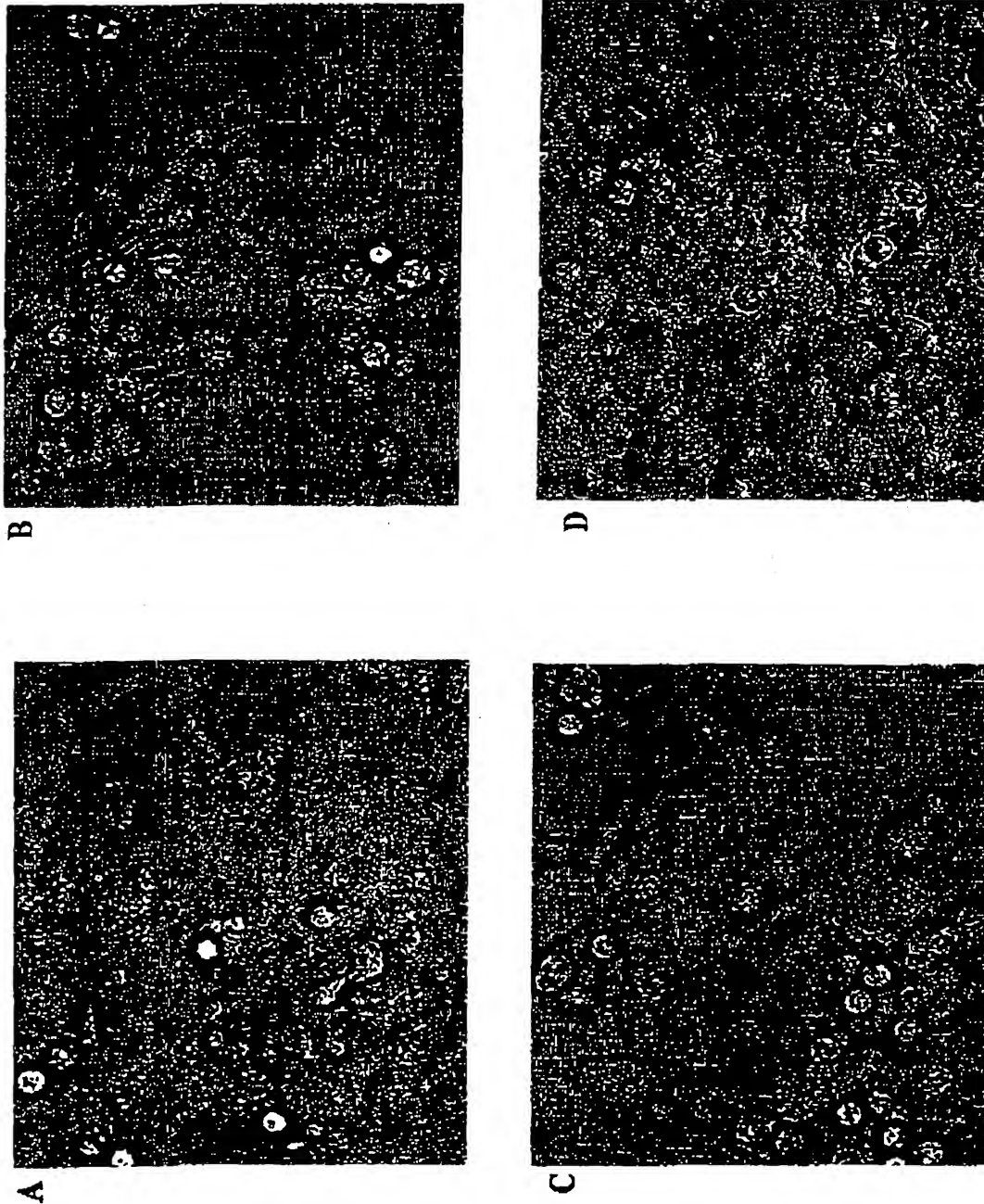
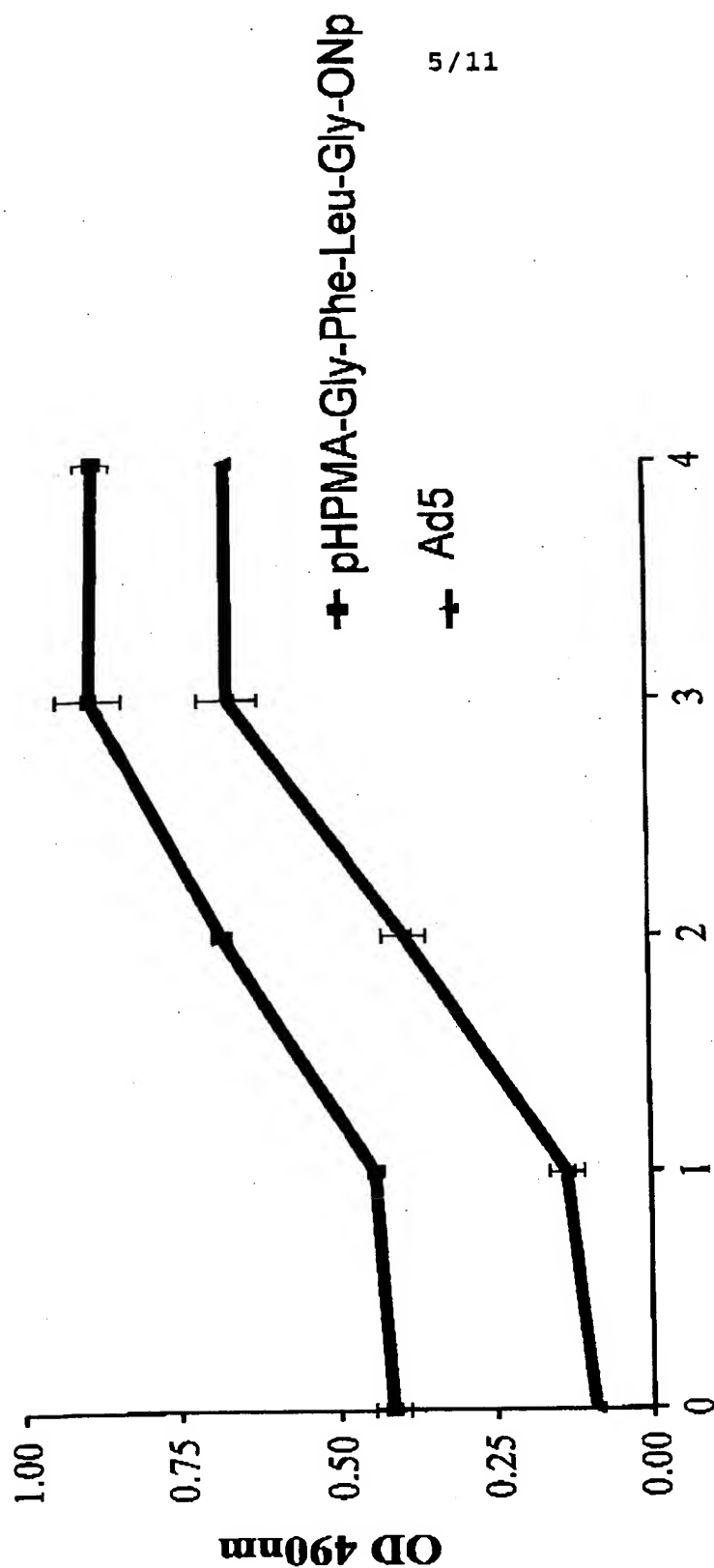


Figure 4. Reduction of cytopathic effect of wt Ad5 by addition of pHPMA-Gly-Gly-ONp



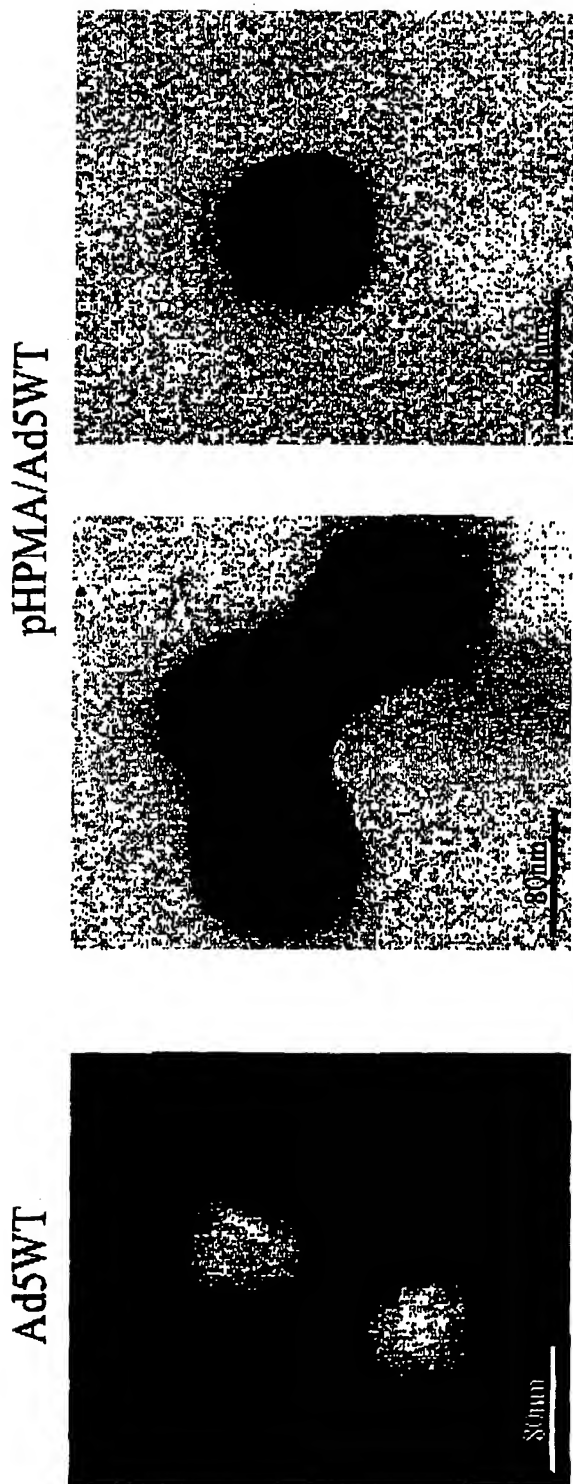
A549 cells (A) were infected with Ad5WT (B), pEG/Ad5 (C) and pHPMA(EC82)/Ad5 (D) at 10^3 particles/cell. 48 hours post-infection cells were examined for presence of cytopathic effect due to adenovirus infection.

Figure 5.



2-fold dilution of virus added to antibodies

Figure 6: Physical Characteristics of pHPMA coated adenovirus



6/11

Ad5WT and pHPMA coated Ad5WT were stained with 2% phosphotungstic acid, pH 7.0 and examined by TEM. Observation by TEM showed the particle size for both Ad5WT and coated virus to be 80nm in diameter; Ad5WT was negatively stained whereas due to the presence of pHPMA, the coated virus was positively stained. Measurement by photon correlation spectroscopy (PCS) showed that the particle size for Ad5WT was the same as that measured by TEM at 80nm. However, when the particle size of pHPMA coated virus was determined by PCS it was shown to be almost 20% greater than that of Ad5WT at 94nm.

7/11

Figure 7

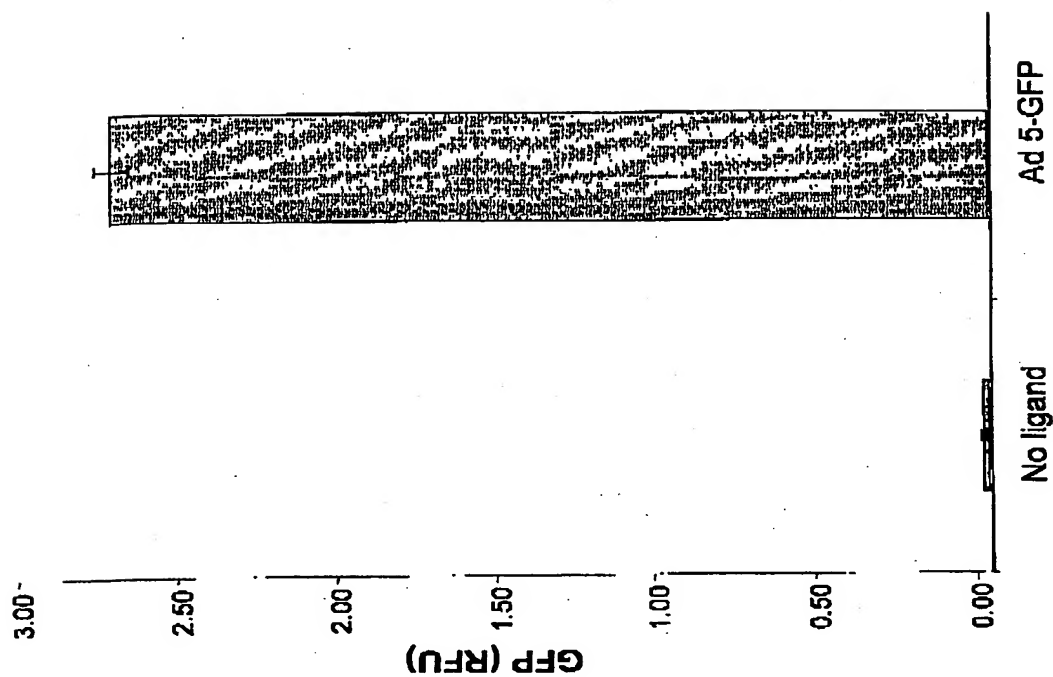
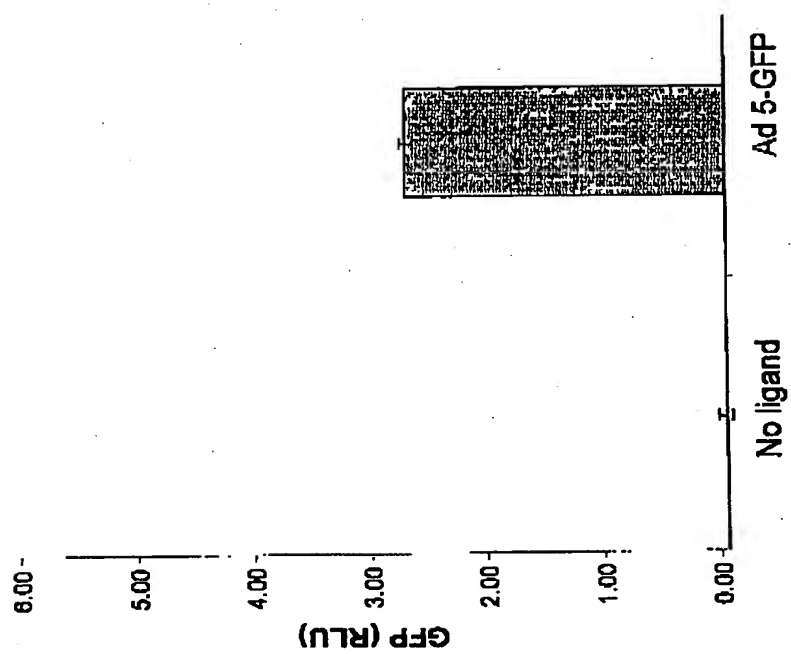
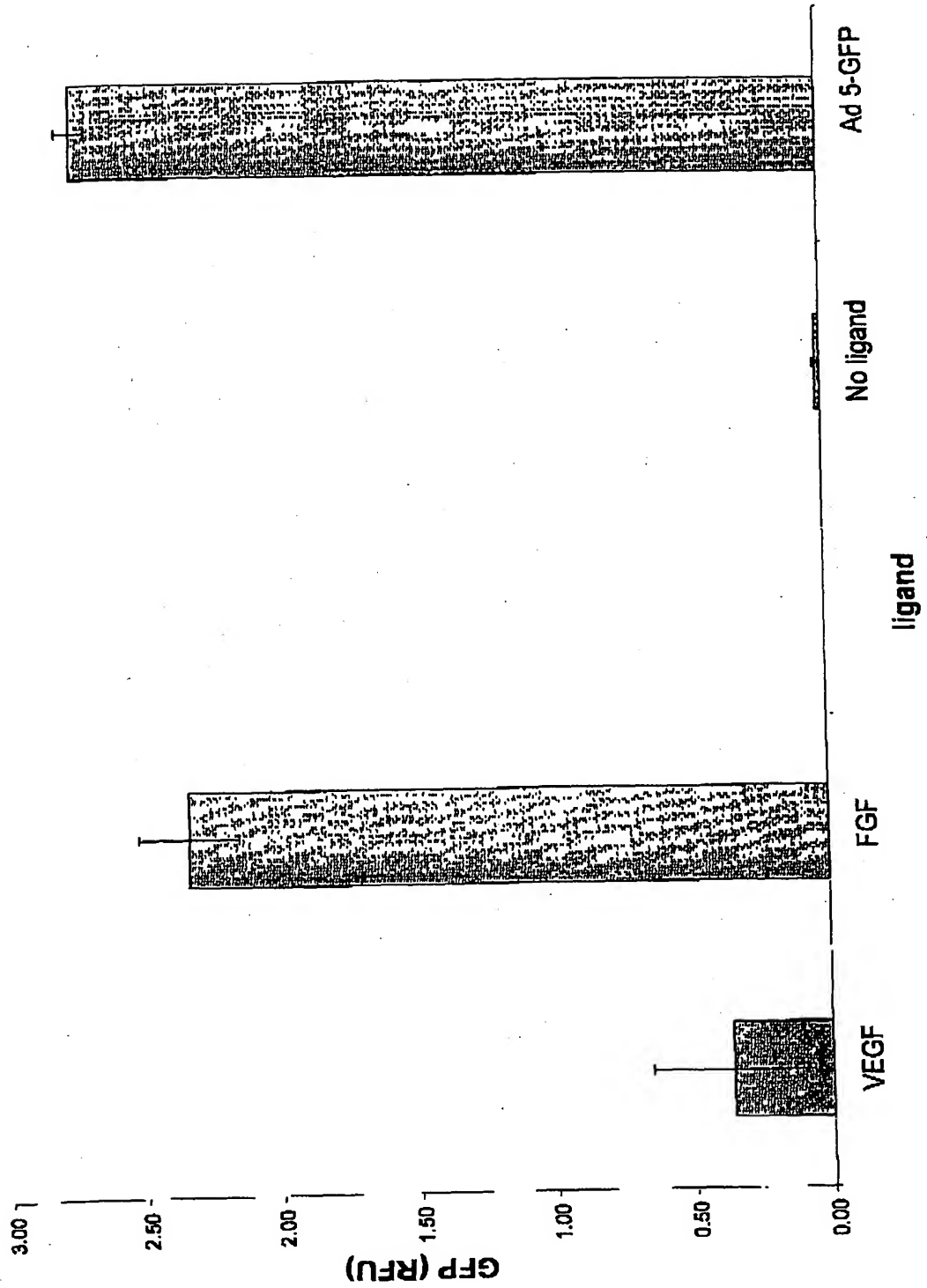


Figure 8



9/11

Figure 9



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Figure 10

Infection of 1×10^5 Human Vein umbilical endothelial cells (HUE cells) with 1×10^8 ($\sim 1 \times 10^6$ pfu) Adenovirus type 5 particles retargeted with ligands via PHPMA-GLY-GLY-ONp

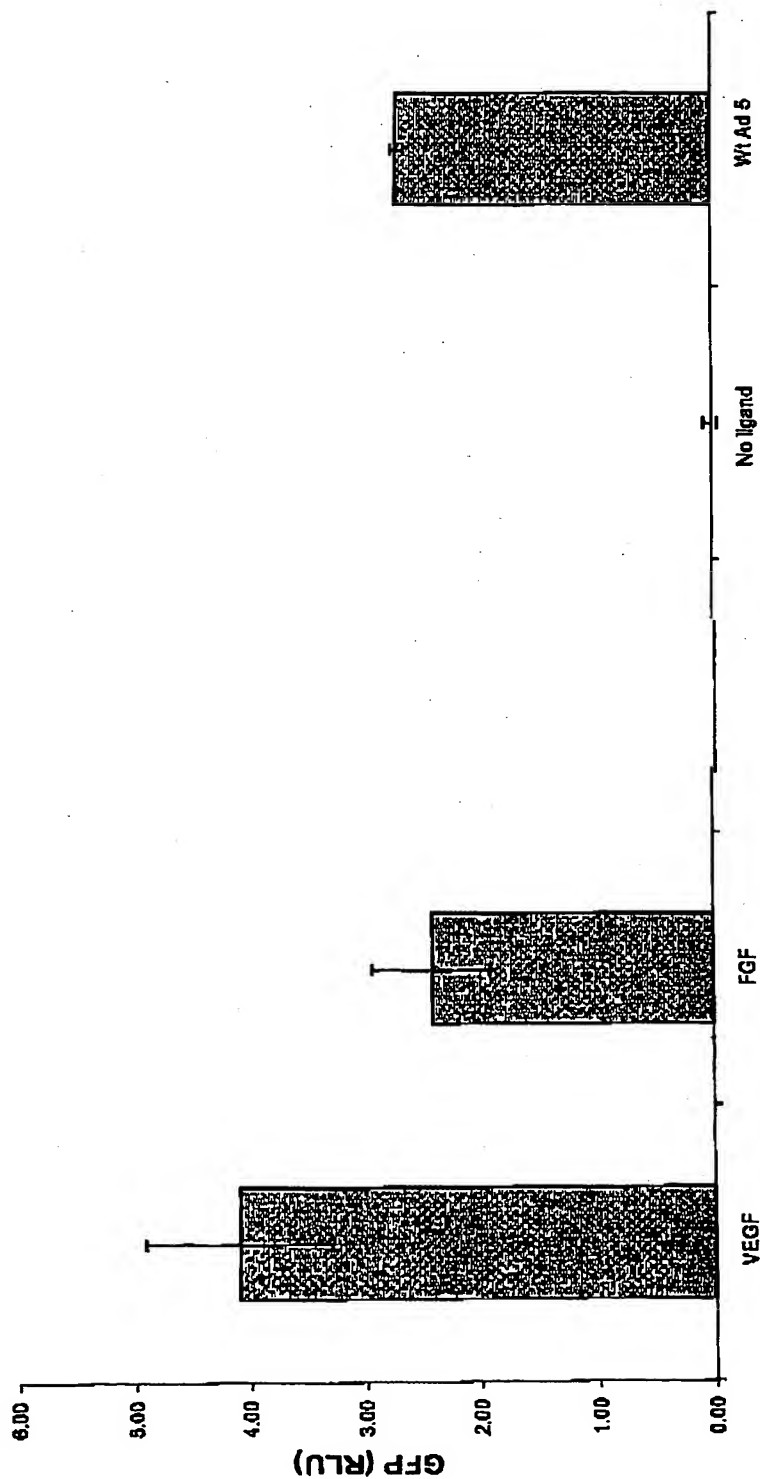
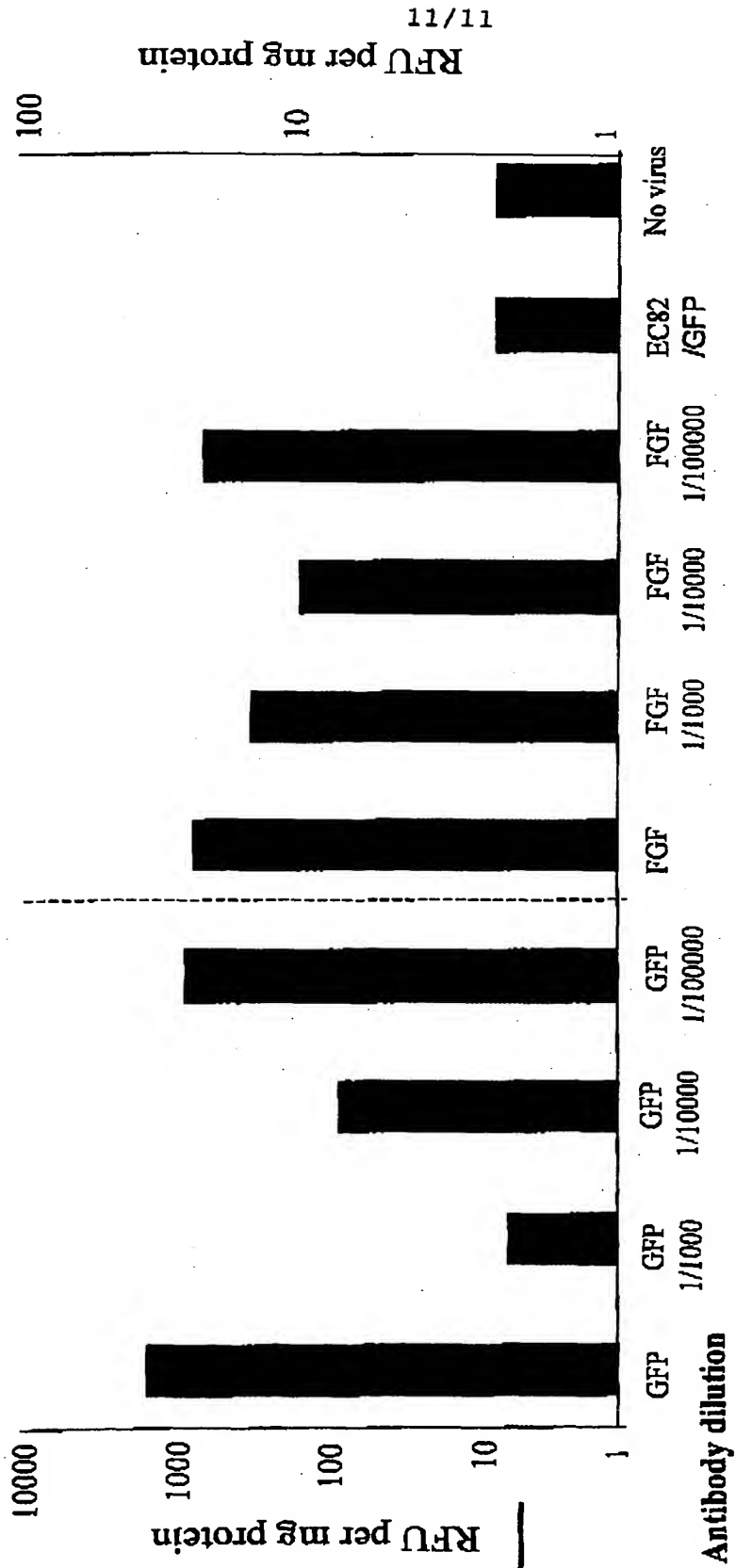


Figure 11



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